



Physiological and biochemical responses of two keystone polychaete species: *Diopatra neapolitana* and *Hediste diversicolor* to Multi-walled carbon nanotubes

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ABSTRACT

Multi-walled carbon nanotubes (MWCNTs) are one of the most important carbon Nanomaterials (NMs). The production and use of these carbon NMs is increasing rapidly and, therefore, the need to assess their presence in the environment and associated risks has become increasingly important. However, limited literature is available regarding the impacts induced in aquatic organisms by this pollutant, namely in invertebrate species. *Diopatra neapolitana* and *Hediste diversicolor* are keystone polychaete species inhabiting estuaries and shallow water bodies intertidal mudflats, frequently used to evaluate the impact of environmental disturbances in these systems. To our knowledge, no information is available on physiological and biochemical alterations on these two species due to MWCNTs exposure. Thus, the present study aimed to assess the toxic effects of different MWCNTs concentrations (0.01; 0.10 and 1.00 mg/L) in both species physiological (regenerative capacity and respiration rate) and biochemical (energy reserves, metabolic activities, oxidative stress related biomarkers and neurotoxicity markers) performance, after 28 days of exposure. The results obtained revealed that exposure to MWCNTs induced negative effects on the regenerative capacity of *D. neapolitana*. Additionally, higher MWCNTs concentrations induced increased respiration rates in *D. neapolitana*. MWCNTs altered energy-related responses, with higher values of electron transport system activity, glycogen and protein concentrations in both polychaetes exposed to this contaminant. Furthermore, when exposed to MWCNTs both species showed oxidative stress with higher lipid peroxidation, lower ratio between reduced and oxidized glutathione, and higher activity of antioxidant (catalase and superoxide dismutase) and biotransformation (glutathione-S-transferases) enzymes in exposed organisms.

1. Introduction

The International Organization for Standardization defines the term nanomaterials (NMs) as the ‘material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale’. The term nanoscale is defined as size range from approximately 1 nm to 100 nm (Potočník, 2011). Due to their nanosize, NMs possess unique mechanical, catalytic, optical and electrical properties (Dowling et al., 2004). Among different types of carbon NMs, fullerenes (nC₆₀), carbon nanotubes (CNTs), and graphene are the most important (Sanchez et al., 2012). CNTs are hollow graphene cylinders that are microns to millimeters in length and they can be

divided in single-walled (SWCNTs) with a diameter of 0.7–3 nm, and multi-walled (MWCNTs) with a diameter of 10–25 nm (Baughman et al., 2002). Chemical vapor deposition, carbon arc discharge, laser ablation, or electrolysis methods, are the common methods used to produce CNTs (Mwangi et al., 2012). The industrial production and the number of applications of CNTs are increasing rapidly due to their important properties such as low mass density, high mechanical strength, high electron/hole mobility, and high thermal conductivity (Baughman et al., 2002). SWCNTs are used in broad industrial and biomedical applications (e.g.: in electronic devices, wastewater treatment and drug delivery systems). MWCNTs are used in catalysis, fillers in composites for antistatic applications, components within recharge-

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able battery electrodes, and composite materials (Du et al., 2013). Considering all these applications, current annual demand for CNTs has already increased from 3700–4100 t to 5300–5720 t in 2015 and is estimated reach 12,000 t in 2020 (Thayer, 2007). Increased industrial production and application of such materials are likely to increase the release of CNTs into the environment, and to inevitably enter aquatic systems through processes such as general weathering, from disposals containing consumer products, accidental spillages, and waste discharges (Nowack and Bucheli, 2007). Due to the difficulty to be dispersed in water (hydrophobic and non-biodegradable characteristics) (Donaldson et al., 1998) these materials can be accumulated by aquatic biota. CNTs bioaccumulation has not been fully evaluated. However, published research on uptake and accumulation of these materials have showed that pathways of uptake are mainly through body surface, digestive and respiratory systems (Jackson et al., 2013). CNTs can enter cells by diffusion causing membrane disruption, protein oxidation, genotoxicity, energy transduction interruption, reactive oxygen species (ROS) formation, and release of toxic constituents (Klaine et al., 2008). Nevertheless, although literature has already demonstrated the toxic effects of CNTs on vertebrate species (Oberdörster et al., 2006; Cheng et al., 2007; Thomas et al., 2011; Chen et al., 2012; Handy et al., 2008; Klaper et al., 2010; Kataoka et al., 2016), less studies have focused on the toxicity on invertebrate species, especially from marine environments (Mwangi et al., 2012; Templeton et al., 2006; Miller et al., 2015; Mesarič et al., 2015). In particular, limited information is available on the effects of these emerging pollutants on polychaetes that are commonly the most abundant group of organisms in marine environments (Rodrigues et al., 2011). In the present study, to assess the effects of CNTs, particularly MWCNTs, two polychaete species were selected, *Diopatra neapolitana* (Delle Chiaie 1841) and *Hediste diversicolor* (O.F. Müller, 1776) which have a key role in the structure and functioning of ecosystems and are recognized as good bioindicators (Dean, 2008). These two species inhabit intertidal mudflats of estuaries and shallow water bodies of the Atlantic and Indian Oceans (Cedex, 1924; Arias and Paxton, 2015) as well as the Mediterranean Sea (Dağlı et al., 2005) where they play an important ecological role (Catalano et al., 2012; Thit et al., 2015) and represent an important economic resource (Costa et al., 2006; Cunha et al., 2005). *H. diversicolor* is one of the most widely used bioindicator species of environmental change, and is commonly used to assess pollution impacts due to metals (Bouraoui et al., 2010; Burlinson and Lawrence, 2007; Pook et al., 2009; Moreira et al., 2006), silver and copper oxide nanoparticles (Buffet et al., 2014; Cong et al., 2014; Thit et al., 2015), polycyclic aromatic hydrocarbons (Catalano et al., 2012; Sun and Zhou, 2008), pharmaceuticals (Pires et al., 2016; Maranhão et al., 2014), pH variation (Freitas et al., 2016a) and salinity shifts (Scaps and Borot, 2000). *D. neapolitana* is also a good bioindicator of metal contamination (Freitas et al., 2012) organic matter enrichment (Carregosa et al., 2014), pharmaceutical drugs (Freitas et al., 2015), salinity shifts and pH decrease (Pires et al., 2015). Nevertheless, studies assessing the toxic effects of carbon NMs induced in polychaetes are unknown. Effects of NMs on biological growth, cellular structure, oxidative stress, signal pathway, proteins and genes in invertebrates species, particularly in polychaetes are still unclear (Mu et al., 2016). For this reason, the present study evaluated the impacts of three different MWCNTs concentrations (0.01; 0.10 and 1.00 mg/L) in polychaetes *D. neapolitana* and *H. diversicolor*. Recently, Sun et al. (2016) developed a customized dynamic probabilistic material flow model (DP-MFA) that predicted concentrations of the most important engineered NMs (including CNTs) in waste streams and environmental compartments in the EU in 2020. Model projections obtained by these authors showed that predicted environmental concentrations of CNTs in surface water will be 0.97 ng/L (mean value for EU in 2020), which is four orders of magnitude lower than the values tested in the present study. Nonetheless toxic effects in the aqueous phase have been observed at mg/L concentrations (Nowack et al., 2013).

Consequently the levels of MWCNTs used in the present study aimed to obtain observable effects and were also selected taking into consideration previous studies conducted using similar range of carbon NMs concentrations on vertebrate (Kataoka et al., 2016) and invertebrate (Alloy and Roberts, 2011; Pretti et al., 2014) species. Therefore, in the present study polychaetes were exposed to MWCNTs for 28 days and the physiological (regenerative capacity of *D. neapolitana*, mortality and respiration rate of both species) as well as biochemical (oxidative stress, metabolic and energy related markers) alterations were assessed. Additionally, neurotoxicity was also investigated by measure of the activity of Cholinesterases (ChE). The activity of ChE has been widely used as a marker of neural changes in marine organisms exposed to pollution (Gomes et al., 2014), but so far and to our knowledge, there are no reported studies on polychaetes ChE activity after CNT exposure.

2. Methods

2.1. Sampling and experimental conditions

D. neapolitana and *H. diversicolor* were collected in the Mira channel, the southern shallow arm of the Ria de Aveiro lagoon (Portugal). Specimens with similar weight (*H. diversicolor*: 0.50 ± 0.2 g; *D. neapolitana*: 2.27 ± 0.61 g) were used to prevent differences on biochemical and physiological responses as well as on MWCNTs accumulation.

In the laboratory, *D. neapolitana* specimens were pushed out from their tubes, and both species were placed in different aquaria for a 20 d acclimation period. Aquaria were filled with a mixture of fine and medium sediment from the sampling area (a clean sampling area with sediment median value 1.59; percentage of fines 6.75 ± 0.79 ; percentage of organic matter content 3.24 ± 0.44) approximately 1/3 of the height of the aquarium, and artificial seawater (salinity 28) set up by mixing artificial sea salt (Tropic Marin Sea Salt) with reverse osmosis (RO) water. During the acclimation period organisms were under constant photoperiod 12 h light: 12 h dark, temperature (18 ± 1 °C) and aeration conditions. To respect species feeding requirements, during this period every two-three days the specimens of *H. diversicolor* were fed *ad libitum* with commercial fish food (48.6% protein and 7.7% fat) (Freitas et al., 2016a), while *D. neapolitana* specimens were fed *ad libitum* with small fragments of frozen cockles (Pires et al., 2012).

To assess the impact of MWCNTs on the regenerative capacity of *D. neapolitana*, immediately before the exposure assay, individuals were removed from their new tubes, anaesthetized with a 4% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ solution, and amputated at the 60th chaetiger under a stereomicroscope (Pires et al., 2012). Posteriorly, both species were exposed to three different MWCNTs concentrations (0.01; 0.10 and 1.00 mg/L) plus control (0.00 mg/L) for 28 days.

For each condition and species, 3 aquaria were used with 10 specimens of *H. diversicolor* and 9 *D. neapolitana* per aquarium (12 aquaria for each species). To ensure that *D. neapolitana* individuals (n=3) used for the regenerative capacity analysis were consistently separated from the individuals used for the biochemical analysis (n=6), a glass divider was used in each aquarium. As for the acclimation period, aquaria were filled with a mixture of fine and medium sediment from the sampling area (approximately 1/3 of the height of the aquarium), and artificial seawater (salinity 28) set up by the addition of artificial sea salt (Tropic Marin Sea Salt) to RO water. During the exposure period organisms were under constant photoperiod 12 h light: 12 h dark, temperature (18 ± 1 °C) and aeration conditions. As for the acclimation period, during the exposure period every two-three days, the specimens of *H. diversicolor* were fed *ad libitum* with commercial fish food (48.6% protein and 7.7% fat) (Freitas et al., 2016a), while *D. neapolitana* specimens were fed *ad libitum* with small fragments of frozen cockles (Pires et al., 2012).

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